APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Jan. 1993, p. 236–242
0099-2240/93/010236-07$02.00/0

Vol. 59, No. 1

Production of Extracellular Proteins by the Biocontrol Fungus *Gliocladium virens*

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Received 4 August 1992/Accepted 5 November 1992

Gliocladium virens **is a common saprophytic fungus that is mycoparasitic on a large number of fungi. Responses of *G. virens* toward its environment were examined by monitoring the presence of extracellular proteins in culture fluid during time course experiments. Culture fluid of *G. virens* grown on glucose, washed cell walls of *Rhizoctonia solani* (one of its hosts), olive oil, or chitin contained β-glucanase, *N*-acetylglucosaminidase, lipase, and proteinase activities. There were relatively minor amounts of other enzymatic activities tested. Levels of extracellular enzyme activity varied with the age of the culture and the substrate used as the carbon source. Substrate-associated differences in enzyme activities were detected as early as 8 h after transfer of mycelia from stationary-phase cultures to fresh media. When *G. virens* was grown on host cell wall material, β-glucanase had the greatest specific activity of any enzyme tested at 8 h. This result suggests that β-glucanase may be the first enzyme important in the *G. virens-R. solani* interaction. Analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis showed that some of the polypeptides were present in the culture fluid at relatively constant amounts and others accumulated early, at intermediate times, or late in the 8-day incubation test period. Several of the polypeptides present in the culture fluid during the first 24 h disappeared completely by 48 h. Consequently, it appears that extracellular proteins in cultures of *G. virens* are regulated by a combination of gene regulation and protein degradation.**

Fungi are nonphotosynthetic microorganisms that obtain nutrition by absorption of metabolites from their environment. When an insoluble substrate is encountered, a fungus must be able to synthesize and secrete appropriate enzymes and absorb enzymatically released, soluble metabolites. Not unexpectedly, a large number of extracellular fungal enzymes have been discovered. Frequently, an individual fungal strain is found to produce a number of enzymes with the same or subtly different activities (11, 29, 38).

Gliocladium virens is a saprophytic and mycoparasitic fungus that has been reported to have biological control activity (10, 22). Many different enzymes have been observed in cultures of *G. virens*. Cellulolytic enzyme production in *G. virens* isolated from jute has been analyzed under fermentation conditions (9, 14). Roberts and Lumsden (26) found that a high-molecular-weight (MW) fraction of a 5-day-old culture of a biocontrol strain of *G. virens* grown on a wheat bran extract medium contained laminarinase, amylase and protease activities and low levels of carboxymethyl cellulase and chitinase activities. They did not detect protease, chitinase, or carboxymethyl cellulase activity and only rarely detected laminarinase and amylase activities in 5-day-old cultures of *G. virens* grown in minimal medium supplemented with *Pythium ultimum* (a host of *G. virens*) tissue.

It is clear that extracellular enzymes of fungal mycoparasites can digest cell walls of their hosts, and several researchers suggest that these enzymes are important for biocontrol activity (5, 6, 15, 35). However, the role of these fungal enzymes in the biocontrol process has not been definitively proven (17, 31). Lack of correlation of lytic enzyme production with biocontrol activity (21), or lack of demonstrable biocontrol activity in high-MW fractions from culture fluid of mycoparasites (26), may be the result of in vitro culturing of the biocontrol agent. Maximal inhibitory activity by a high-MW fraction may require a particular combination of enzymes at certain relative concentrations for effective hydrolytic activity. These conditions may be ephemeral in laboratory-grown cultures. Consequently, we investigated the extracellular proteins produced by *G. virens* grown in minimal medium containing various carbon sources. We simultaneously monitored a number of enzymatic activities in cultures of *G. virens* over an 8-day period and examined the extracellular polypeptides produced by the fungus when it was grown on purified substrates or cell wall material of one of its hosts. The extracellular polypeptides were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the relative concentrations of some of these polypeptides were determined.

MATERIALS AND METHODS

Fungal strain and media. *G. virens* RV14 is a clone of *G. virens* GV-P (ATCC 52045) reported to have biocontrol activity against *Rhizoctonia solani* (10). The parent culture has been stored in 10% glycerol at −70°C since 1983, and it was used for the development of a transformation system (32). Conidia from the parent culture were streaked onto potato dextrose agar. After 5 to 9 days, conidia were suspended in sterile H_2O and used to inoculate modified Murashige and Skoog medium (MSM) at a final concentration of 10^4 conidia per ml. The non-nitrogen-containing salts of MSM were not modified (19); however, we used ammonium sulfate (1.9 g/liter) as the nitrogen source and glucose (24 g/liter) as the carbon source and supplemented the medium with thiamine (85 μg/liter).

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TABLE 1. p-NP substrates used in screening enzymatic activity in fluid from *G. virens* cultures

p-NP conjugate	Activity detected	EC no.	Reference(s)
N-Acetyl-β-D-galactosaminide	β-*N*-Acetylhexosaminidase	3.2.1.52	20
N-Acetyl-β-D-glucosaminide	*N*-Acetyl-β-glucosaminidase (chitobiase)	3.2.1.30	36
β-D-Cellobioside	Cellulose 1,4-β-cellobiosidase	3.2.1.91	13
β-D-Fucopyranoside	β-D-Fucosidase	3.2.1.38	32
β-D-Galactopyranoside	β-Galactosidase	3.2.1.23	13, 32
α-D-Glucopyranoside	α-Glucosidase	3.2.1.20	12, 34
β-D-Glucopyranoside	β-Glucosidase	3.2.1.21	1, 24, 33
	Endo-1,3(4)-β-D-glucanase	3.2.1.6	3
	Glucan 1,3-β-glucosidase	3.2.1.58	35
α-D-Mannopyranoside	α-Mannosidase	3.2.1.24	18
β-D-Xylopyranoside	Xylan 1,4-β-xylanxylosidase	3.2.1.37	11, 23
Palmitate	Triacylglycerol lipase	3.1.1.3	8

Culture conditions. The fungus was incubated with shaking (100 rpm) for 120 h at 25°C. The fungal tissue was separated from the culture fluid by centrifugation (10,000 × *g*, 5 min) and washed once by centrifugation with an equal volume of sterile MSM containing no carbon source. The fungal tissue was resuspended in an equal volume of MSM with one of the following carbon sources at 1 g/liter replacing glucose at 24 g/liter: glucose (MSMg), washed cell walls of *R. solani* (MSMr), olive oil (MSMo), or colloidal chitin (MSMc). The washed cell walls were prepared from a 10-day-old still broth culture of *R. solani* LU7. After incubation at 100 rpm and 25°C, the culture fluid of *G. virens* was separated from the fungal tissue by filtration through a 10-μm-mesh nylon net. Protein concentration was determined with Coomassie Plus Protein Assay Reagent (Pierce) in a 300-μl microtiter plate assay; the optical density (OD) at 460 nm was subtracted from the OD at 595 nm. Protein concentrations were calculated from the observed OD values by standard curves (for bovine serum albumin) with a quadratic regression fit (2).

The extracellular macromolecules in the culture fluid were concentrated in a MacroSep centrifugal concentration device with an MW cutoff of 10,000 (Filtron). The high-MW fraction was washed with 10 mM MES (morpholineethanesulfonic acid; pH 6.0) and concentrated again. The samples were kept cold, and the protein concentration was adjusted to 0.5 or 1.0 mg/ml. One portion of each sample was diluted with an equal volume of glycerol and stored at −20°C, and another portion was diluted with an equal volume of SDS-isoelectric focusing-PAGE solubilization buffer {10 mg of CHES (2-[*N*-cyclohexylamino]ethanesulfonic acid; pH 9.5) per ml, 20 mg of SDS per ml, 10% glycerol, 5% β-mercaptoethanol}, denatured for 10 min at 100°C, and stored at −70°C.

Enzyme assays. The *p*-nitrophenol (p-NP) conjugates used in this study and enzyme activities detected are listed in Table 1. The activity observed in our high-MW fractions is the sum of the activities of an undetermined number of enzymes capable of cleaving the substrate tested. Generally, enzymes with exoenzyme activity or a preference for oligomeric substrates are most active on p-NP substrates. However, within a given EC class, differential hydrolysis of some of these substrates has been reported (33). Azocoll has been used to detect nonspecific proteolytic activity (e.g., see reference 27).

Initial assays of high-MW fractions were performed within 48 h of sample collection. No loss of enzymatic activity was detected for at least 30 days in samples processed as described above. The amount of protein added to each enzyme assay was adjusted to yield OD readings below 2.5. Substrates containing p-NP conjugates were used at 1 mM in 200-μl assay mixtures in microtiter plates, and Azocoll was used at 5 mg/ml in 400-μl assay mixtures in microcentrifuge tubes. The assay mixtures were buffered with 50 mM sodium citrate (pH 3.0, 4.5, or 6.0), with 50 mM sodium MOPS (morpholinepropanesulfonic acid; pH 7.5), or with the universal borate-citrate-sodium phosphate buffer of Carmody (4) at pH 2.5 to 10.0 with a gradient of 0.5 pH units. All assay mixtures were incubated for 60 min at 25°C. For the p-NP assays, Tris HCl (pH 9.0) was added to 167 mM in a final volume of 300 μl, and the OD of the released p-NP was read at 410 nm. For the Azocoll assay, the assay mixture was centrifuged (12,000 × *g*, 4 min, 4°C); 300 μl of the supernatant was transferred to a microtiter plate, and its OD was read at 550 nm.

The E_{401} (1 mM) for our p-NP standard was verified and correlated to readings obtained under our assay conditions on a microtiter plate reader at 410 nm. Units of enzyme activity against the p-NP substrates are reported in micromoles of p-NP released under our assay conditions per milliliter of culture fluid. Proteinase activity is expressed in arbitrary units, and thus it is not directly comparable to the activity observed against the other substrates.

Electrophoresis. One-dimensional SDS-PAGE was performed by standard techniques (16) with 5% acrylamide stacking and 12% acrylamide separating gels (180 by 180 by 0.75 mm). Each lane was loaded with 5 μg of protein, and polypeptides in gels were visualized by color silver staining (28). The gels were analyzed with a BioImage Visage 110 system (Millipore).

Experiments were repeated two times. For protein production and enzymatic activity, the major variation among experimental replications was the time of onset of observed changes rather than the shape of the curve. Presumably, variation in the time of onset of response resulted from lag time differences in the cultures. Consequently, representative as opposed to averaged results are presented. Multiple SDS-PAGE separations were performed on each sample.

RESULTS

Changes in the culture fluid pH and protein concentration when *G. virens* was grown on MSM with different carbon sources are shown in Fig. 1. The pH of the filtered culture fluid dropped to below 4 during the first 8 h after stationary-phase cultures of the fungus were transferred to fresh media and then rose during the next several days. Protein concentration increased rapidly over the first 3 days.

Enzyme assays. Preliminary experiments showed that a pH

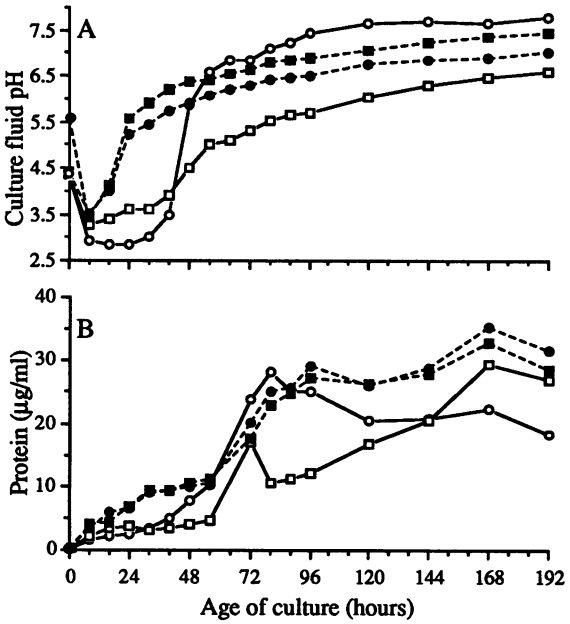

FIG. 1. Culture fluid pH (A) and concentration of soluble proteins (B) in cultures of *G. virens* grown in MSM supplemented with glucose (○), washed cell walls of *R. solani* (●), olive oil (□), or chitin (■).

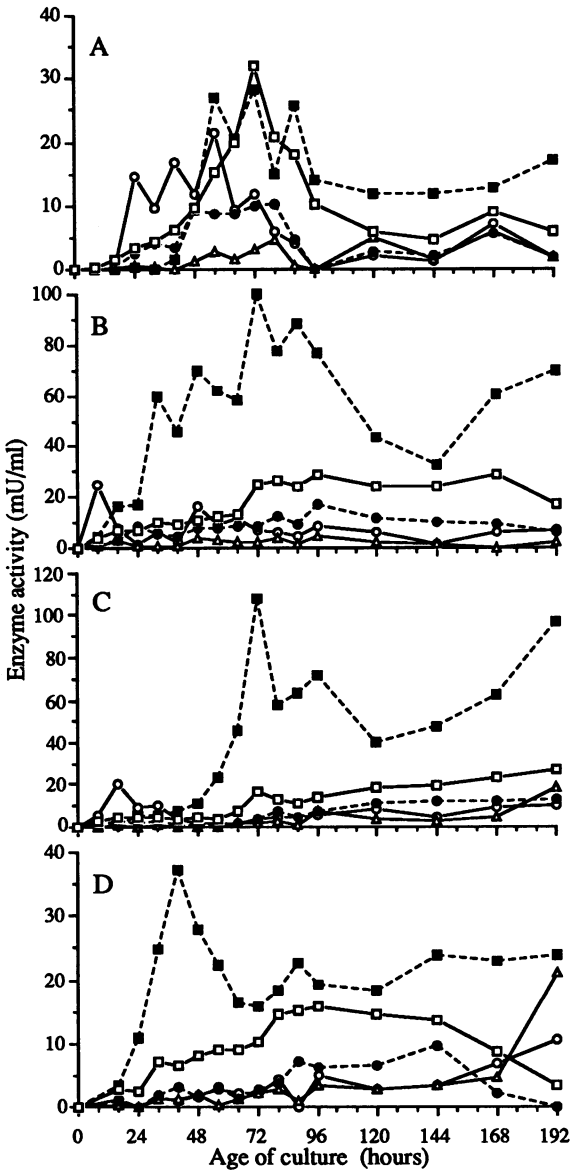

FIG. 2. Hydrolytic activity against several p-NP conjugates in high-MW culture fractions collected at various times. *G. virens* RV14 was grown in MSMg (A), MSMr (B), MSMo (C), and MSMc (D). Symbols: ○, p-NP-β-D-cellobioside; ●, p-NP-β-D-xylopyranoside; □, p-NP-α-D-glucopyranoside; ■, p-NP-β-D-galactopyranoside; △, p-NP-β-D-fucopyranoside.

of 4.5 was optimal for screening all enzymatic activities except proteinase activity. All of the substrates listed in Table 1 were used in time course experiments (assayed at pH 4.5). Hydrolytic activity was observed against all substrates except p-NP-α-D-mannopyranoside. There was relatively little activity against several of the substrates (Fig. 2), and these substrates were not used in subsequent studies. The major enzymatic activities were further tested over a wide pH range in order to observe potential shifts in pH optima for enzyme activity over time. When *N*-acetylglucosaminidase activity was tested at pH 2.5 to 10.0, the maximum activity was observed at pH 5.5 (Fig. 3). When *G. virens* was grown on MSMg, MSMo, or MSMc, acid *N*-acetylglucosaminidase activity occurred at 24 h but later declined relative to the activity observed at higher pH. Figure 3 understates the prominence of the acid activity at 24 h because the activity is expressed in units per milliliter and there was comparatively little protein in fluid from younger cultures. Representative specific activities against p-NP-*N*-acetyl-β-D-glucosaminide in MSMc were 614 U/mg (pH 2.5) and 583 U/mg (pH 5.5) at 24 h, 175 U/mg (pH 2.5) and 380 U/mg (pH 5.5) at 72 h, and 214 U/mg (pH 2.5) and 478 U/mg (pH 5.5) at 144 h.

Activity against p-NP-*N*-acetyl-β-D-glucosaminide paralleled activity against p-NP-*N*-acetyl-β-D-galactosaminide. The average of the ratio of activity against p-NP-*N*-acetyl-β-D-glucosaminide to activity against p-NP-*N*-acetyl-β-D-galactosaminide in all samples was 1.670, with a standard deviation (SD) of 0.217. Thus, the latter activity is not presented separately in a figure. The ratio of p-NP-β-D-galactopyranoside-hydrolyzing activity to p-NP-β-D-glucopyranoside-hydrolyzing activity did vary considerably among samples. The greatest variation was observed in samples from cultures grown on MSMg; the ratio was 0.841 (SD = 1.535). The other ratios were as follows: MSMr, 0.406 (SD = 0.166); MSMo, 0.454 (SD = 0.356); and MSMc, 0.357 (SD = 0.146). We will refer to the hydrolysis of p-NP-β-D-glucopyranoside as β-glucanase activity.

No shifts in pH optima were observed in enzymatic activity against either p-NP-β-D-glucopyranoside or p-NP palmitate; therefore, only data from cultures of RV14 grown on MSMr are presented (Fig. 4). The β-glucanase activity had a pH optimum of 4.5, and the lipase activity had a broad pH optimum range between pH 3.5 and 7.5. Under our assay conditions, p-NP palmitate was somewhat unstable, spontaneously releasing some p-NP above pH 8.5. The high-MW fraction that was added in these assays appeared to have a

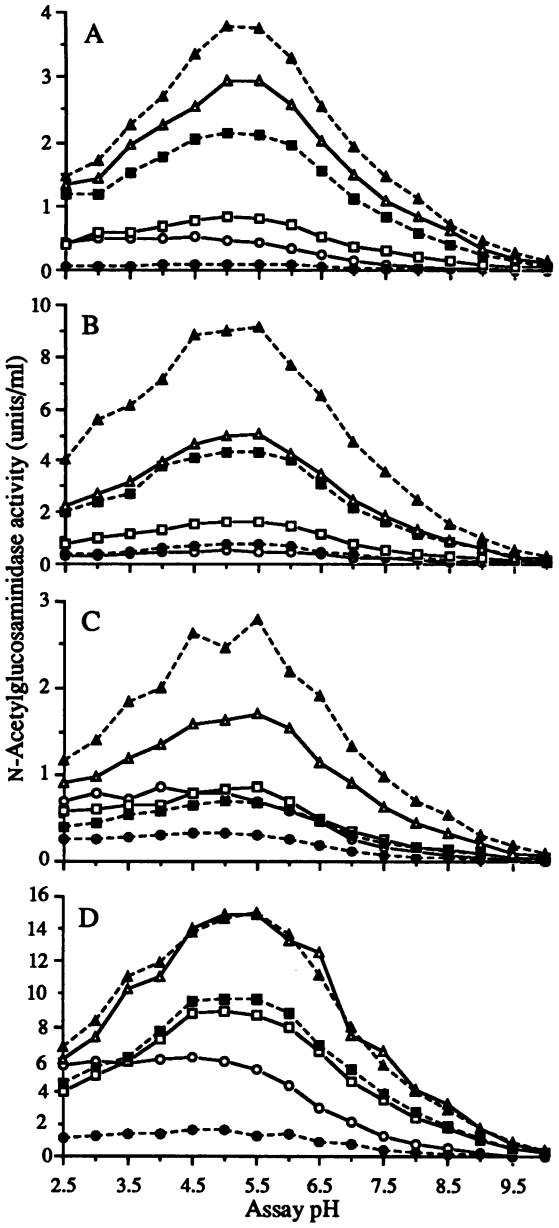

FIG. 3. *N*-Acetylglucosaminidase activity in high-MW fractions from cultures of *G. virens* at various ages tested over a range of pH. The culture media used were MSMg (A), MSMr (B), MSMo (C), and MSMc (D). Symbols: ○, 24 h; ●, 48 h; □, 72 h; ■, 96 h; △, 120 h; ▲, 144 h.

stabilizing effect on p-NP palmitate, yielding negative values after the no-enzyme control values at pH 9.5 and 10.0 were subtracted. Values less than 0 are not shown in Fig. 4.

There was acid proteinase activity (pH optimum, 4.5) in young cultures of *G. virens*. At 24 h, there was approximately 2 U/ml in cultures grown on MSMg or MSMo, and this activity disappeared by 48 h. In samples from MSMr or MSMc cultures, there was 5 U of proteinase activity per ml (pH 4.5) at 24 h; this activity decreased to 2 U/ml by 48 h and disappeared by 72 h. By 48 h, proteinase activity with a pH optimum of 7.5 predominated in all cultures (Fig. 5).

For the major hydrolytic enzyme activities, the maximum

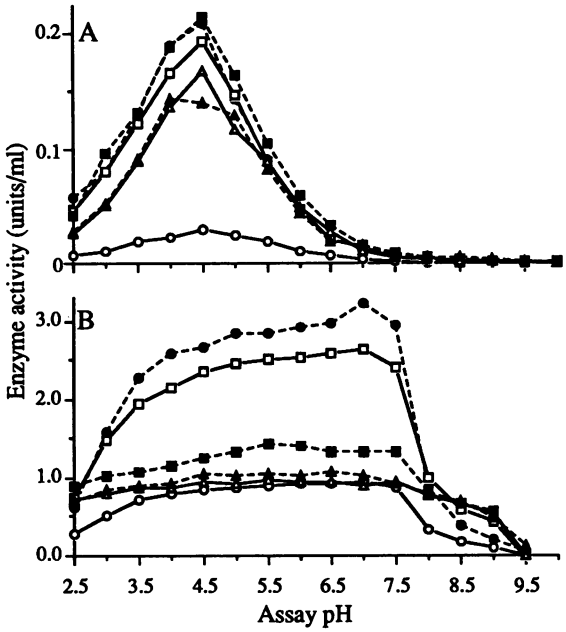

FIG. 4. Activity against p-NP-β-D-glucopyranoside (A) and p-NP palmitate (B) in high-MW fractions from cultures of *G. virens* grown in MSMr. Symbols: ○, 24 h; ●, 48 h; □, 72 h; ■, 96 h; △, 120 h; ▲, 144 h.

activity and the incubation time at which it was achieved varied with respect to the carbon source in the growth medium (Table 2). Cultures of *G. virens* grown on MSMr maintained maximum activity against p-NP-β-D-glucopyranoside from 2 to 4 days. Cultures grown on MSMc had two peaks of activity against p-NP-*N*-acetyl-β-D-glucosaminide: 6 U/ml at 24 h (dropping to 1.5 U/ml by 40 h) and a maximum of 14 U/ml at 7 days.

Specific activity relates the amount of enzyme activity to the total protein present. Thus, it is a measurement of the relative prominence of an enzyme activity in crude culture fluid. The maximum specific activity against p-NP-β-D-glucopyranoside occurred at 8 h, and the maximum activity against p-NP-β-D-glucosaminide occurred at 192 h (Fig. 6). Both of these activities were detected as early as 8 h, but increases in these activities were initially not as great as the increases in activities of other proteins in the culture fluid,

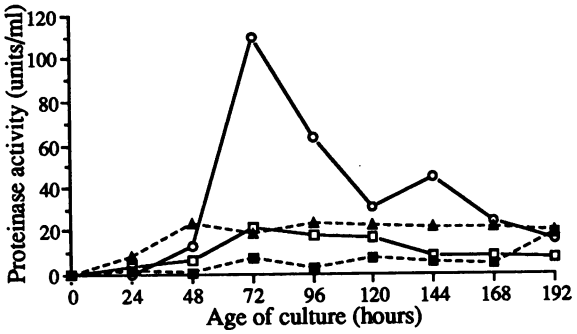

FIG. 5. Proteinase activity tested at pH 7.5 in high-MW fractions from cultures of *G. virens* grown in MSM supplemented with glucose (○), washed cell walls of *R. solani* (□), olive oil (■), or chitin (▲).

TABLE 2. Major hydrolytic activity in cultures of *G. virens* grown on various carbon sources

Substrate[a]	Maximum activity in U/ml (age of culture in days) in:			
	MSMg	MSMr	MSMo	MSMc
p-NPG	0.25 (3)	0.2 (3)	0.15 (4)	0.1 (4)
p-NPAGA	5 (7)	8 (8)	6 (8)	14 (7)
p-NP palmitate	2 (3)	2 (3)	4.5 (3)	2 (2)
Azocoll	115 (3)	22 (4)	15 (8)	22 (2)

[a] p-NPG, p-NP-β-D-glucopyranoside; p-NPAGA, p-NP-*N*-acetyl-β-D-glucosaminide.

resulting in a drop in specific activity. Total activity against p-NP-β-D-glucopyranoside remained relatively constant after 48 h (Fig. 4), whereas activity against p-NP-*N*-acetyl-β-D-glucosaminide continued to increase throughout the experiment (Fig. 3). Therefore, when the amount of culture fluid protein stopped increasing rapidly (after 3 days), the specific activity against p-NP-*N*-acetyl-β-D-glucosaminide increased.

Polypeptide electrophoresis. Figure 7 is a contrast-enhanced photograph of color silver-stained extracellular polypeptides from cultures of *G. virens*. Younger cultures showed greater differences in polypeptide content among samples grown on different substrates than did older cultures. Overall, between 20 and 38 polypeptide bands were resolved in each sample by PAGE. The complexity of the samples (measured by the number of bands resolved) did not correlate with the carbon source used. Use of the color silver staining procedure provides evidence in addition to MW that certain polypeptides in different samples are the same polypeptide. Many of the polypeptides among different samples could be putatively identified as being identical by electrophoretic and staining characteristics. Consequently, the change in integrated OD of these polypeptides over time could be quantitated. Some of the polypeptides were present in the culture fluid at relatively constant amounts, while others accumulated early, at intermediate times, or late in the 8-day incubation period (Fig. 8).

DISCUSSION

It is essential to look at the complex of extracellular enzymes in order to fully understand the response of a fungus to its environment. Many extracellular enzymes work

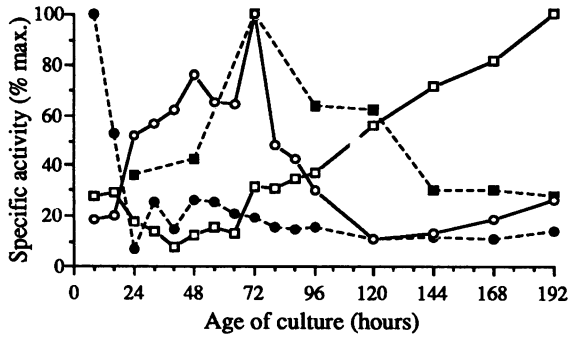

FIG. 6. Percentage of maximum specific activity against four substrates in high-MW fractions from cultures of *G. virens* grown in MSMr. Symbols: ○, p-NP palmitate; ●, p-NP-β-D-glucopyranoside; □, p-NP-*N*-acetyl-β-D-glucosaminide; ■, Azocoll.

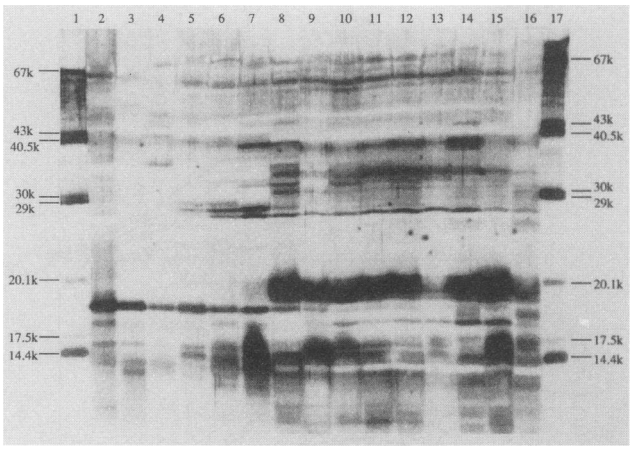

FIG. 7. SDS-PAGE separation of extracellular polypeptides from cultures of *G. virens*. Lanes: 1 and 17, MW markers (in thousands); 2, MSMg (16 h); 3, MSMo (16 h); 4, MSMc (16 h); 5, MSMr (16 h); 6 through 13, MSMr (24 to 192 h at 24-h intervals); 14, MSMc (192 h); 15, MSMo (192 h); 16, MSMg (192 h).

in concert for the digestion of macromolecules. For example, complete digestion of cellulose normally requires the activity of three enzymes (EC 3.2.1.4, EC 3.2.1.21, and EC 3.2.1.91). A mycoparasite is presented with an even greater challenge than producing a number of enzymes for the digestion of a single type of macromolecule because the host tissue that it encounters is a complex matrix comprising a mixture of macromolecules. In particular, the cell walls of *R.*

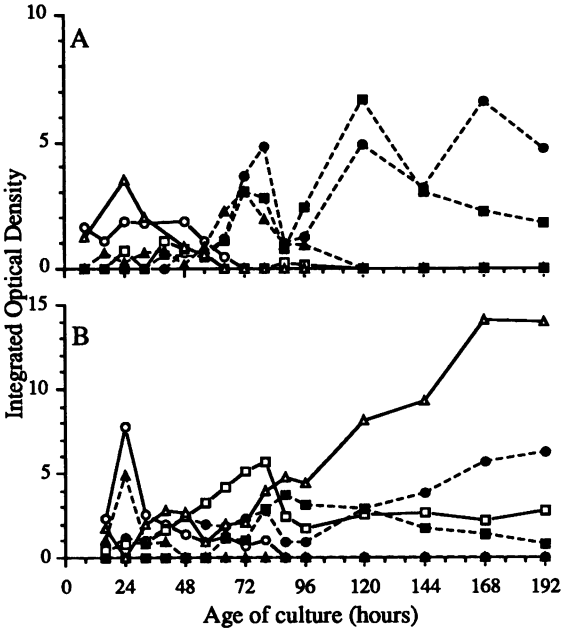

FIG. 8. Analysis of polypeptide accumulation in culture fluid of *G. virens* RV14 grown on MSMr (A) or MSMc (B). MWs of polypeptides (separated by SDS-PAGE) are as follows: those in common in both media, 39,600 (○), 32,400 (●), 16,200 (□), and 6,900 (■); those unique to MSMr cultures (A), 35,500 (△) and 27,800 (▲); and those unique to MSMc cultures (B), 38,200 (△) and 28,500 (▲).

solani are composed of a matrix of chitin, β-glucans, glycoproteins, and lipids (7, 30).

Our experimental approach was designed to simultaneously monitor a number of activities sampled over time in crude enzyme preparations, allowing us to more fully investigate enzymatic responses that the fungus makes to its environment. With the exception of the Azocoll, the substrates that were used are dimers or oligomers containing p-NP. The use of p-NP conjugates allowed us to test as many as 10 substrates (and controls) on eight samples in a single microtiter plate. This experimental approach permits a number of conclusions to be drawn from our observations even though crude, unpurified samples were used. Glucose in the culture medium did not preclude the production of hydrolytic enzymes, which indicates that *G. virens* makes detectable levels of enzymes for the digestion of macromolecules even though the substrates are absent. With the exception of α-D-mannopyranoside, activity against all enzymatic substrates tested was observed regardless of the carbon source used in the culture. However, substrate-associated differential enzyme activities were detected as early as 8 h after transfer of mycelia from a stationary-phase culture to fresh medium. This result shows that the fungus was capable of a very rapid response to its environment considering the steps involved, i.e., transition from stationary growth phase, recognition of insoluble substrates, induction of mRNA synthesis (assuming regulation at the transcriptional level), protein synthesis, export of the proteins from the cell through the periplasm to the culture fluid, and accumulation of the enzymes to detectable levels. The substrate-associated differences that we observed were, for the most part, differences in the timing and levels of accumulation of specific enzymatic activities. These observations suggest that there is intricate regulation of the individual components in the complex of extracellular enzymes.

Since the pH of the culture fluid changed over the incubation period, it was considered possible that enzymes with different pH optima are present at different times. There was evidence of increased acid *N*-acetyl-β-glucosaminidase activity relative to the activity at higher pH in 24- to 48-h-old cultures of *G. virens* when glucose, olive oil, or chitin was used as the carbon source (Fig. 3). The acid *N*-acetyl-β-glucosaminidase activity was not as pronounced when washed cell walls of *R. solani* were used. This attenuation may be the result of the higher initial pH (Fig. 1), or the complex composition of the cell wall material may attenuate the formation of the acid *N*-acetyl-β-glucosaminidase activity. Higher levels of acid proteinase activity (with maximum activity at pH 4.5) were observed in young cultures grown in MSMr and MSMc than in cultures grown in either MSMg or MSMo. Other than these, there was no evidence of pH optimum shifts of the major enzymatic activities during culture of *G. virens* on the various substrates. These data suggest that different enzymes which have *N*-acetyl-β-glucosaminidase or proteinase activity were produced at different times and that if the fungus produced multiple forms of either β-glucanases or lipases, they had similar pH activity optima.

The level of enzymatic activity varied with the age of the culture and the substrate used as the carbon source for *G. virens*. When *G. virens* was grown on medium containing washed cell walls of *R. solani*, maximum lipase and proteinase activities overlapped with the maximum β-glucanase activity (3 to 4 days), while *N*-acetylglucosaminidase activity was still increasing at the end of the experiment (8 days). The maximum levels of enzyme activity in MSMr cultures were intermediate with respect to cultures containing single carbon sources. The timing of enzyme accumulation may be suggestive of the order in which *G. virens* encounters the individual components in the cell wall matrix, and the amount of enzyme produced may reflect the lower concentrations of the individual components of the washed cell walls relative to the concentrations of the purified substrates in the media. At 8 h, β-glucanase had the greatest specific activity of any enzyme tested in culture fluid of *G. virens* grown on MSMr. This observation indicates that it is one of the first enzymes produced in response to cell wall material and that it may be the first enzyme important in the *G. virens-R. solani* interaction.

Several of the polypeptides present in the culture fluid during the first 24 h disappeared completely by 48 h (Fig. 7). The appearance of these early polypeptides may reflect a carryover of protein synthesis from the previous culture conditions (before the fungal tissue was washed and transferred to fresh MSM with various carbon sources). These polypeptides seem not to be induced; rather, their synthesis may not be immediately down-regulated by the new culture conditions. Some of these polypeptides were present in the stationary-phase culture fluid from which the fungal tissue was transferred, supporting the idea of carryover synthesis (data not shown). However, substrate-specific protein synthesis was also induced as early as 8 h (shown by the presence of enzymatic activity).

The concentrations of some of the polypeptides were relatively constant, while others disappeared from the culture fluid at various incubation times (Fig. 7 and 8). Thus, it appears that there are specific mechanisms allowing differential enzyme degradation. Mechanisms that may be involved include production of specific proteinases capable of digesting certain classes of extracellular proteins, differential glycosylation, and differences in amino acid sequences that result in variation in protein stability. We are currently investigating these potential mechanisms. The intricate regulation of extracellular proteins in cultures of *G. virens* appears to be achieved by a combination of gene regulation and protein degradation.

REFERENCES

1. **Acebal, C., R. Arche, M. P. Castillon, P. Estrada, and D. Romero.** 1988. β-Glucosidase production by *Trichoderma reesei* QM9414 on wheat straw: location and some kinetic features. Acta Biotechnol. **8:**341–348.
2. **Bearden, J. C.** 1978. Quantitation of submicrogram quantities of protein by an improved protein-dye binding assay. Biochim. Biophys. Acta **533:**525–529.
3. **Bielecki, S., and E. Galas.** 1991. Microbial β-glucanases different from cellulases. Crit. Rev. Biotechnol. **10:**275–304.
4. **Carmody, W. R.** 1961. An easily prepared wide range buffer series. J. Chem. Educ. **38:**559–560.
5. **Chet, I., A. Ordentlich, R. Shapira, and A. Oppenheim.** 1990. Mechanisms of biocontrol of soil borne plant pathogens by rhizobacteria. Plant Soil **129:**85–92.
6. **Elad, Y., I. Chet, and Y. Henis.** 1982. Degradation of plant pathogenic fungi by *Trichoderma harzianum*. Can. J. Microbiol. **28:**719–725.
7. **Farkas, V.** 1990. Fungal cell walls: their structure, biosynthesis and biotechnological aspects. Acta Biotechnol. **10:**225–238.
8. **Feller, G., M. Thiry, J. L. Arpigny, and C. Gerday.** 1991. Cloning and expression in *Escherichia coli* of three lipase-encoding genes from the psychrotrophic Antarctic strain *Moraxella* Ta144. Gene **102:**111–115.
9. **Gomes, J., I. Gomes, H. Esterbauer, W. Kreiner, and W. Steiner.** 1989. Production of cellulases by a wild strain of *Gliocladium virens*: optimization of the fermentation medium and partial characterization of the enzymes. Appl. Microbiol.

Biotechnol. **31:**601–608.
10. **Howell, C. R.** 1982. Effect of *Gliocladium virens* on *Pythium ultimum*, *Rhizoctonia solani* and damping-off of cotton seedlings. Phytopathology **72:**496–498.
11. **John, M., and J. Schmidt.** 1988. Xylanases and β-xylosidase of *Trichoderma lignorum*. Methods Enzymol. **160:**662–671.
12. **Kelly, C. T., M. Giblin, and W. M. Fogarty.** 1986. Resolution, purification, and characterization of two extracellular glucohydrolases, α-glucosidase and maltase, of *Bacillus licheniformis*. Can. J. Microbiol. **32:**342–347.
13. **Kohring, S., J. Wiegel, and F. Mayer.** 1990. Subunit composition and glycosidic activities of the cellulase complex from *Clostridium thermocellum* JW20. Appl. Environ. Microbiol. **56:**3798–3804.
14. **Labudova, I., D. Czajkowska, M. Hayn, W. Steiner, and H. Esterbauer.** 1989. Investigations on the physiology, mutagenesis and cellulase production of *Gliocladium virens*. J. Biotechnol. **12:**123–134.
15. **Labudova, I., and L. Gogorova.** 1988. Biological control of phytopathogenic fungi through lytic action of *Trichoderma* species. FEMS Microbiol. Lett. **52:**193–198.
16. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
17. **Lynch, J. M.** 1989. Environmental potential of the *Trichoderma* exocellular enzyme system. ACS Symp. Ser. **399:**608–618.
18. **Matta, K. L., and O. P. Bahl.** 1972. α-Mannosidase from *Aspergillus niger*. Methods Enzymol. **28:**749–755.
19. **Murashige, T., and F. Skoog.** 1962. A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol. Plant. **15:**473–497.
20. **Ohtakara, A.** 1988. Chitinase and β-*N*-acetylhexosaminidase from *Pycnoporus cinnabarinus*. Methods Enzymol. **161:**462–470.
21. **Ordentlich, A., Q. Migheli, and I. Chet.** 1991. Biological control activity of three *Trichoderma* isolates against *Fusarium* wilts of cotton and muskmelon and lack of correlation with their lytic enzymes. J. Phytopathol. (Berlin) **133:**177–186.
22. **Papavizas, G. C.** 1985. *Trichoderma* and *Gliocladium*: biology, ecology, and potential for biocontrol. Annu. Rev. Phytopathol. **23:**23–54.
23. **Poutanen, K., and J. Puls.** 1988. Characteristics of *Trichoderma reesei* β-xylosidase and its use in the hydrolysis of solubilized xylans. Appl. Microbiol. Biotechnol. **28:**425–432.
24. **Rapp, P.** 1989. 1,3-β-Glucanase, 1,6-β-glucanase and β-glucosidase activities of *Sclerotium glucanicum*: synthesis and properties. J. Gen. Microbiol. **135:**2847–2858.
25. **Ridout, C. J., J. R. Coley-Smith, and J. M. Lynch.** 1986. Enzyme activity and electrophoretic profile of extracellular protein induced in *Trichoderma* spp. by cell walls of *Rhizoctonia solani*. J. Gen. Microbiol. **132:**2345–2352.
26. **Roberts, D. P., and R. D. Lumsden.** 1990. Effect of extracellular metabolites from *Gliocladium virens* on germination of sporangia and mycelial growth of *Pythium ultimum*. Phytopathology **80:**461–465.
27. **Saksirirat, W., and H. H. Hoppe.** 1991. Secretion of extracellular enzymes by *Verticillium psalliotae* Treschow and *Verticillium lecanii* (Zimm) Viegas during growth on uredospores of the soybean rust fungus *Phakopsora pachyrhizi* Syd. in liquid cultures. Phytopathol. Z. **131:**161–173.
28. **Sammons, D. W., and L. Adams.** 1987. Color silver staining of polypeptides in polyacrylamide gels. ACS Symp. Ser. **335:**91–101.
29. **Sharma, S., D. K. Sandhu, and P. S. Bagga.** 1991. Physical characterization of isozymes of endo-β-1,4-glucanase and β-1,4-glucosidase from *Aspergillus* species. FEMS Microbiol. Lett. **79:**99–104.
30. **Sivan, A., and I. Chet.** 1989. Degradation of fungal cell walls by lytic enzymes of *Trichoderma harzianum*. J. Gen. Microbiol. **135:**675–682.
31. **Sreenivasaprasad, S., and K. Manibhushanrao.** 1990. Antagonistic potential of *Gliocladium virens* and *Trichoderma longibrachiatum* to phytopathogenic fungi. Mycopathologia **109:**19–26.
32. **Thomas, M. D., and C. M. Kenerley.** 1989. Transformation of the mycoparasite *Gliocladium*. Curr. Genet. **15:**415–420.
33. **Tochikura, T., K. Sakai, T. Fujiyoshi, T. Tachiki, and H. Kumagai.** 1986. p-Nitrophenyl glycoside-hydrolyzing activities in Bifidobacteria and characterization of β-D-galactosidase of *Bifidobacterium longum* 401. Agric. Biol. Chem. **50:**2279–2286.
34. **Todorovic, R., S. Grujic, J. Kandrac, and M. Matavulj.** 1989. Some properties of β-glucosidase from *Gliocladium virens* C_2R_1. Biotechnol. Appl. Biochem. **11:**459–463.
35. **Ulhoa, C. J., and J. F. Peberdy.** 1991. Regulation of chitinase synthesis in *Trichoderma harzianum*. J. Gen. Microbiol. **137:**2163–2169.
36. **Vazquez de Aldana, C. R. V., J. Correa, P. San Segundo, A. Bueno, A. R. Nebreda, E. Mendez, and F. del Rey.** 1991. Nucleotide sequence of the exo-1,3-β-glucanase-encoding gene, *EXG*1, of the yeast *Saccharomyces cerevisiae*. Gene **97:**173–182.
37. **Vyas, P., and M. V. Deshpande.** 1989. Chitinase production by *Myrothecium verrucaria* and its significance for fungal mycelia degradation. J. Gen. Appl. Microbiol. **35:**343–350.
38. **Waksman, G.** 1991. Purification and characterization of two endo-β-1,4-D-glucanases from *Sclerotinia sclerotiorum*. Biochim. Biophys. Acta **1083:**49–55.